V. EFFECT OF MULTIPLE DOSES OF ANAPHYLATOXIN

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SYNOPSIS

It was manifestly impossible to study the effects of multiple doses as long as the anaphylatoxin possessed a relatively feeble action. The lethal dose of the toxic serum, as prepared by previous workers, was usually about 3 c.c. per 200 gm. of guinea-pig. Doerr[1] stated that the smallest lethal dose known of guinea-pig anaphylatoxin was 2 c.c., tho 1.5 c.c. had been observed to kill in 1 hour. It has been shown in the work reported here that it is possible, with agar, to render guinea-pig serum toxic so that it is acutely fatal in dose of less than 1 c.c. Notable exceptions to Doerr's statement were made by Jobling and Petersen[2] and by Bronfenbrenner,[3] who were able, respectively, to toxify guinea-pig serum so that it killed in dose of 0.31 and 0.5 c.c. The development of a speedy method whereby it was possible to prepare a toxic rat serum the lethal dose of which was but 0.2 to 0.25 c.c., opened the subject to investigation.

It seemed essential to the understanding of the problem of specific anaphylaxis that the behavior of anaphylatoxin be studied under the conditions mentioned. The mystery of the acute specific shock was largely due to the lack of definite facts regarding the amount and nature of the poison concerned. Occasional recoveries, when large amounts of antigen were given at the second injection, would at times

[1] Kolle and Wassermann's Handb. d. pathogen. Mikroorganismen, 1913, 2, p. 1105.
[2] Jour. Exper. Med., 1914, 19, p. 485.
[3] Ibid., 21, p. 480.

be interpreted as due to excessive poison-production, a conclusion implying that a large amount of poison was less toxic than a small amount. It was therefore of interest to ascertain whether there was any difference between the toxic effects when one and when a number of lethal doses were administered. Incidentally, it was hoped that some light would be thrown upon the relation of the dose of anaphylatoxin to the noncoagulability of blood.

The experiments which were made in this direction led to some striking results, not only as regards coagulation, but also as to the effect of large doses in guinea-pigs and especially in rats. The information thus acquired became of extreme value in attacking the problem of anaphylaxis.

The rat anaphylatoxin was prepared by the sol-gel method, using Mixture 6, which yields maximal toxicity in the shortest possible time. After incubating the iced mixture for 15 minutes, a small portion was removed, centrifugated, and tested; if acute death followed the injection of 0.25 c.c. of the serum, the entire mixture was at once centrifugated and the serum used for the experiment. Usually, incubation for 15 minutes gave the desired lethal dose, but if such was not the case, the mixture was retested after a further incubation of 15 minutes. In order to avoid any decrease in toxicity through possible reversion changes the sera were used as soon as prepared. There is no reason to believe, however, that the anaphylatoxin varies appreciably when kept for a short time at room temperature.

The injection, when small doses were administered, took not over 20 seconds. The difference in time between the end of such injection and the last nasal twitch was taken as the time necessary to kill. In several instances, the injections were made slowly but steadily, extending over 2 to 4 minutes, and in such cases the time necessary to kill is expressed in 2 figures; the first of these represents the lapse of time from the end of injection, the second from the start to the final nasal twitch. The speed of the injection, it will be shown, has a distinct influence on the coagulability of the blood.

INJECTION OF GUINEA-PIGS

The results of these experiments are summarized in Table 57. It should be stated that for these tests, 9 different toxic pools were employed, all possessed of an initial lethal dose of 0.25 c.c. It is indeed possible that some may have killed in dose of 0.2 c.c. One pool served for Tests 1, 2, 4, 5, and 13; another for 3, 12, and 14; another for 8 and 15; another for 9 and 16, while separate pools were used for each of the remaining tests. The injections, as usual, were intravenous.

It is seen in Table 57 that tho the number of lethal doses ranged from 1 to 40, the death point was essentially the same in all. In other words, the symptom complex of acute anaphylactic asphyxia requires a

fairly constant time regardless of the number of toxic doses employed. The type of the shock, however, varies distinctly with the number of lethal doses. With moderate amounts, the usual typical symptoms of oncoming dyspnea, spasms, and violent convulsions are noted; with the larger doses the overwhelming effect is such that the convulsions and even spasms are missed, the picture being that of an intense quiet shock. In such case, the animal when released lies limp, with respiration all but suppressed, and shows at most a few tremors of the feet. Incidentally, it may be said that this type of shock is often met with when injecting large amounts of normal sera, cyanid, peptone, etc.

TABLE 57

EFFECT OF MULTIPLE LETHAL DOSES OF ANAPHYLATOXIC SERUM ON GUINEA-PIGS

Guinea-Pig		Serum		Result	Clotting Time in Test Tube (min.)
No.	Weight	c.c. (intravenously)	Lethal Doses		
1	177	0.25	1	3′40″ Typical shock	5
2	182	1.25	5	3′15″ " "	11
3	190	1.25	5	3′ 5″ " "	6
4	190	2.5	10	3′40″ " "	8
5	205	"	"	3′50″ " "	11
6	188	"	"	3′ 5″ " "	
7	192	"	"	3′ " "	
8	208	"	"	3′15″ Quiet shock	8
9	215	"	"	2′40″–5′ " "	11
10	192	5.0	20	3′ Typical shock	
11	205	"	"	4′20″ Quiet shock	0
12	175	"	"	2′55″ " "	
13	210	10.0	40	3′50″–4′25″	25
14	213	"	"	3′55″–4′25″	17
15	205	"	"	1′55″–4′	⊕
16	206	"	"	0′15″–4′15″	⊕
17	200	"	"	1′10″–4′25″	⊕

⊕ Incoagulable.

In all cases, the autopsy, made from 3 to 4 minutes after death, revealed the same typical picture: maximal distention of the lungs and, usually, in animals which had received 10 or more lethal doses, enormous petechiæ in the lungs; heart beating and blood perfectly fluid without sign of clot. The absence of clot after injection of the largest doses of this serum, is all the more remarkable since large doses of rabbit anaphylatoxin (6 c.c.) almost invariably produce rapid clotting.

Effect on Coagulation.—In all but 4 of the animals, an in-vitro test of the coagulability of the blood was made. For this purpose, before cutting open the heart, about 0.5 c.c. of the blood was withdrawn by means of a syringe and transferred to a small test tube (8 to 9 mm. in diameter); observations were then made every minute thereafter. The time needed to form a solid clot (++++) is indicated in the table.

The transfer to the glass considerably accelerated the clotting process, the blood in the heart being often found perfectly fluid for some minutes after the coagulation had taken place in the test tube.[4]

The coagulation time, as given in the table, refers to the time which elapsed after withdrawal of the blood from the heart, this being done 3 minutes after death. When several transfers of blood were made from the heart, for example at 3, 4, and 5 minutes after death, these portions gave a varying coagulation time; that for the second portion was less while that for the third was nearly the same as for the first. More extended observations are desirable, but those made are suggestive of the 'courbe oscillante' of DeWaele.[5]

Of special interest are the tests in which 10 c.c. of serum were injected. In Test 14, in which the injection took 30 seconds, the blood coagulated in the test tube in 17 minutes; in Test 13 an almost equally rapid injection gave a blood which formed a full, but very soft, clot in 25 minutes. On the other hand, in the last three of the series, the same amount of serum being administered but very slowly, the blood was almost incoagulable. This state is indicated in the table by the sign ⊕. In Tests 15 and 16, a very minute clot (+), about 2 mm. in diameter, which could have been easily overlooked, was detected in 4 and 10 minutes, respectively, but the process went no further. In No. 17 the clot became a trifle larger in 12 minutes (++), but this also was arrested; the serum which had been injected into this guinea-pig had been heated to 50 C. for half an hour with the expectation of weakening its thrombin content and thus securing a minimal clot. The heating process evidently had the opposite effect. With the appearance of the minute clot mentioned the blood seemed to increase in viscosity, but this condition soon passed away, the blood becoming as fluid as before.

In Test 11, in which the injection time was short (not recorded), there was no sign of coagulation for 3 hours; the observation was then discontinued until the following morning, when a solid coagulum was found. In No. 9, the injection was given in divided doses, 0.5 c.c. every half minute, but this did not have any perceptible effect either on the time of death or on the coagulation. With the smaller doses (Nos. 1 to 9) the coagulation was slightly retarded, requiring about 2 to 4 times as long as normal blood.

[4] Delayed coagulation of the blood in asphyxia is a well-known fact. Friedberger (Ztschr. f. Immunitätsf., 1910, 8, p. 263) found that the blood of guinea-pigs, killed by blow or compression, did not coagulate in 15 minutes; that obtained after death from injection of anaphylatoxin also remained free from clot for from 10 to 20 minutes. These figures presumably refer to the intracardial condition.

[5] Ztschr. f. Immunitätsf., 1913, 17, p. 314.

This method of testing the coagulability of the blood is capable of improvement, especially in view of the fact that the withdrawal of blood from the heart through a needle, introduces a factor of contact which can be avoided. The heart pipet method, which was eventually time of death or on the coagulation. With the smaller doses (Nos. 1 (Parts VII and IX), might be expected to give a strictly noncoagulable blood, were it applied to guinea-pigs injected with large multiple doses of anaphylatoxin.

The conclusion to be deduced from these tests is that rat anaphylatoxin when injected tends to retard either directly or indirectly the coagulation process. The very slow injection of a large dose may give a practically noncoagulable blood.

The In-Vitro Anticoagulant Action of Anaphylatoxin.—As shown in Table 57, the blood of guinea-pigs injected with anaphylatoxin, when transferred to a test tube, shows some retardation of coagulation. If this was due to the mere presence of the poison, it should be possible to secure a similar retardation, if not complete incoagulability, by making an in-vitro mixture of anaphylatoxic serum and fresh normal blood, and the results of a large number of experiments made with this object in view certainly indicated a marked inhibitory action.

One method of procedure was to place 0.25 c.c. of the rat anaphylatoxin in a small test tube and then to add 1 or 2 volumes of blood, drawn as rapidly as possible from the heart. The two were mixed and the fluid was then examined every minute until solid coagulation took place. A better method seemed to be to draw up the blood, either from the exposed heart or from the jugular vein, into a syringe containing anaphylatoxin. By pulling back the piston, the two fluids were quickly mixed, and the mixture was then at once discharged into a test tube. In these tests care was taken to employ an anaphylatoxin which was fatal in dose of 0.25 c.c.

Normal guinea-pig blood, drawn from the heart, clots in 4 minutes or less, but if a series of punctures is made on the same heart, through the pleural wall, extreme variations in coagulability will be encountered. Thus, in a series of 10 consecutive punctures, the range was from 1 minute to incoagulability. This fact made it plain that satisfactory results could be obtained only when the blood was drawn from an exposed heart or vein.

In a test according to the latter method, 0.25 c.c. of jugular blood plus 0.5 c.c. of anaphylatoxin coagulated in 14 minutes, while a mixture of equal volumes clotted in 7 minutes. This test was repeated a number of times with essentially the same result. Rabbit blood seemed to

be retarded by rat anaphylatoxin to a less extent than guinea-pig blood, while rat blood was apparently still less delayed.

There is no doubt but that the rapid admixture of anaphylatoxin with normal blood considerably delays coagulation; the larger the amount of the former the more marked is this result. Control tests, in which the blood was drawn into a syringe containing 1 or 2 volumes of normal rat serum, invariably coagulated in about 1 minute.

INJECTION OF WHITE RATS

The behavior of multiple doses of anaphylatoxin in the guinea-pig having been studied, it was next in order to extend the observations to the white rat, which apparently had never been used for this purpose. It may be stated, however, that Ritz and Sachs[6] found that white mice were not killed by anaphylatoxin, but were killed by peptone injections. Since the mice had died of specific anaphylactic shock, it was inferred that more poison was produced in vivo than had been injected, or else that the mouse-serum anaphylatoxin differed from that of the guinea-pig. It was therefore an interesting question as to what would be the behavior of the rat in response to one or more doses of the homologous anaphylatoxin. The tests made with that object in view revealed a remarkable example of natural immunity or tolerance of the poison.

The rat anaphylatoxin was prepared in the same way as that used for the guinea-pigs. Five different pools were used. One of these with a lethal dose of 0.25 c.c. was employed for the first 6 tests given in Table 58; another pool of the same strength was used for Tests 9 and 10. The lethal dose of the serum used for Test 11 was 0.2 c.c. That for No. 7 was a trifle weak, 0.25 c.c. giving a near-kill; the number of lethal doses injected into No. 7 was therefore between 10 and 20. The serum used for No. 8 also failed to kill in 0.25-c.c. dose and hence the number of lethal doses was between 20 and 40. The failures to kill in these titrations may be simply indicative of individual resistance.

The injections were all intravenous; at first the caudal vein was used, but it was soon found that the femoral vein was more convenient. The injection time of the first 6 tests was short, probably from 5 to 20 seconds according to the dose. With the larger dose (Nos. 8 to 11) the time was respectively ¾, 1¾, 1⅓, and 1 minute.

Even a cursory examination of Table 58 shows that the rat possesses an extraordinary tolerance of the homologous anaphylatoxin. The injection of 20 guinea-pig lethal doses had practically no effect. With 40 of such lethal doses, some respiratory disturbance and slight

[6] Centralbl. f. Bakteriol., R., 1912, 50, Beiheft, p. 45; i. e., Bericht üb. d. 5te Tagung d. Fr. Ver. f. Mikrobiol. in Dresden, 1911.

transitory jerks were noted. With 75 doses the result was more severe but recovery was prompt.

It is to be noted that Rat 11, which received 75 guinea-pig lethal doses, actually was injected with a volume of serum corresponding to 10% of its body weight. A normal serum if given to a guinea-pig in dose representing 5% of its body weight will almost invariably kill. In this case an intensely toxic serum was given in twice that amount without causing death. After the injection of 15 c.c. of the toxic serum the rat was at first slightly excited, then became prone and respiration gradualy faded to nil; after about half a minute it slowly returned, and within 2 minutes the rat had apparently recovered. By contrast, Rat 9, which received 10 c.c. of the serum (75 per kilo), showed slight jerks while being injected but none thereafter; the respiration was slightly increased and the animal was quiet.

The anaphylatoxin injected into Rat 11 was fatal to a guinea-pig of 247 gm. in dose of 0.25 c.c. in 3 minutes 20 seconds; that is to say, it was toxic in 1 c.c. per kilo of guinea-pig. The dose of 15 c.c. given to the rat represents 100 c.c. per kilo of rat. Weight for weight, the rat can tolerate more than 100 times as much anaphylatoxin as the guinea-pig. This fact is all the more remarkable when it is realized that the rat serum, in a given time, can yield an anaphylatoxin which is easily 10 times more toxic than that of the guinea-pig. If it is assumed that the plasma is equally labile, it will be seen that the rat would be extremely susceptible to shock were it not for some natural protection. The tolerance noted is therefore suggestively teleologic. It will be shown that the rabbit likewise shows a tolerance for anaphylatoxin.

TABLE 58

EFFECT OF MULTIPLE GUINEA-PIG LETHAL DOSES OF HOMOLOGOUS ANAPHYLATOXIC SERUM ON WHITE RATS

Rat		Serum		Result
No.	Weight	c.c. (intravenously)	Doses	
1	110	0.25	1	Nil
2	115	0.5	2	"
3	119	1.25	5	"
4	110	1.25	5	"
5	135	2.0	8	Increased respiration
6	145	2.5	10	Nil
7	120	5.0	10–20	"
8	125	10	20–40	"
9	133	10	40	Slight
10	155	10	40	"
11	151	15	75	Severe
12	158	15 (g.p.s.)	7.5–10	Increased respiration

Several explanations of this tolerance can be readily formulated. For instance, the resistance might be assumed to reside in the cells of the body, the cytoplasm offering extreme resistance to dislocation; or, it could be imagined that the plasma of the blood and of the cells exerted a destructive action which could be conceived as oxidative, lytic, or reversible. The action of the blood on the poison is one that is open to experimentation. By means of the transfusion method it should be possible to find out how long the injected poison remains in the circulation. Unfortunately, but one experiment of this kind was made; still it suffices to show that the poison disappears from the circulation of the rat in less than 15 minutes.

Rat 11, which had received 75 guinea-pig lethal doses, was selected for the transfusion experiment, the method of work being the same as that used in connection with the experiments recorded in Table 65, Part VI. After the rat had apparently completely recovered from the effects of the injection, 2 c.c. of blood were drawn from the heart and injected intravenously into a guinea-pig of 178 gm. The result was nil; there was no sign of respiratory disturbance or spasm, and at most a very slight peripheral irritation. The total time which elapsed from the end of the injection into the rat to the end of the injection into the guinea-pig was 15 minutes, while the transfer time from the moment the blood entered the syringe until it was injected into the recipient was 25 seconds. Hence, it follows that in the 'reaction time' of 14 minutes 35 seconds, the time within the body of the rat, much of the poison had disappeared.

On the supposition that the rat contained 10% of its body weight of blood (15 c.c.), and that the volume of the blood after the injection of 15 c.c. of serum had not decreased, it follows that 30 c.c. of the mixed blood represented 75 lethal doses of poison. Consequently, the 2 c.c. transfused, should contain 5 lethal doses; the results show that it actually had little or none. This fact certainly indicates a rapid removal of the poison from the circulation of the rat. That the blood of this animal was not quite normal is seen in this, that immediately after the transfusion, 0.5 c.c. of blood drawn from the heart and transfused to a small test tube began to clot in 4 minutes and became solid in 5. Ordinarily, rat blood will coagulate in 1 or 2 minutes.

Heterologous Anaphylatoxin.—In the first 11 tests given in Table 58, the rats were injected with the homologous or rat anaphylatoxin. It was possible that the rat could change or destroy the homologous

poison more readily than the heterologous; the latter might in that case be poisonous for the rat. To test this point, an anaphylatoxin was prepared by the sol-gel method with guinea-pig serum. A preliminary test showed that 2 c.c. were fatal to a guinea-pig of 200 gm. in 3 minutes 15 seconds, and that 1.5 c.c. gave a near-kill. Fifteen cubic centimeters of this anaphylatoxin, representing therefore from 7.5 to 10 guinea-pig lethal doses, when injected into Rat 12 had no effect. Clearly, the tolerance of the rat holds for the heterologous as well as for the homologous poison.

INJECTION OF RABBITS

Friedberger and Castelli[7] tested the effect of antisheep serum from immune rabbits on normal rabbits and found that the latter showed a distinct tolerance for the poison which was present in such serum. Of a serum which was toxic in dose of 0.5 c.c. per 200 gm. of guinea-pig, 9 c.c. were injected into a rabbit of 770 gm. without producing any effect; this represents 18 guinea-pig lethal doses, or 4.7 per 200 gm. of rabbit. In another test a rabbit of 1600 gm. received 10 c.c. of a serum which was fatal in dose of 0.16 c.c. per 200 gm. of guinea-pig; this represents 62.5 guinea-pig lethal doses, or 7.8 per 200 gm. of rabbit.

Doerr and Weinfurter[8] obtained a similar result. In the one test which they made with an antisheep serum they gave a rabbit of 800 gm. 5 c.c. of a serum which was fatal in dose of 0.1 c.c. per 200 gm. of guinea-pig; this corresponds to 50 guinea-pig lethal doses, or to 12.5 per 200 gm. of rabbit. It will be seen from these tests that weight for weight the rabbit can tolerate more than 12 guinea-pig lethal doses.

Inasmuch as the toxicity of the antisheep serum must be ascribed to the anaphylatoxin which is made in vivo in the rabbit, it is evident that the rabbit must possess a marked tolerance for the poison. In view of this fact it was to be expected that an injection of an anaphylatoxin prepared in vitro by the action of agar on rat serum would be equally tolerated. The experiment confirmed this reasoning.

A toxic rat serum, having a lethal dose of 0.25 c.c., was injected intravenously into 2 young rabbits. Rabbit 1 of 585 gm. was given 5 c.c., and Rabbit 2 of 595 gm. 10 c.c. of this agar anaphylatoxin without the slightest effect being produced in either case. The dose per kilo was therefore 8.5 and 16.8 c.c. respectively, while 1.25 c.c. was the

[7] Ztschr. f. Immunitätsf., 1910, 6, p. 267.
[8] Centralbl. f. Bakteriol., I, O., 1912, 63, p. 420.

lethal dose per kilo of guinea-pig. Weight for weight, therefore, the rabbits tolerated from 6.8 to 13.4 times as much poison as would kill a guinea-pig.

Instead of 13.4 guinea-pig lethal doses per 200 gm. of rabbit, it would be of interest to test 50 or more doses in order to ascertain the full extent of the tolerance possessed by the rabbit. The blood and serum of rabbits treated with sheep corpuscles can become very toxic as is well known. The amount of anaphylatoxin present in such treated animals may be large as will be seen from the following observation. A rabbit of 1720 gm. gave a serum which caused acute fatal shock in 200-gm. guinea-pigs in dose of 0.05 c.c. If it is assumed that 10% of the body weight is blood, this would give 172 c.c., representing 103 c.c. of serum. This amount of serum would mean that the rabbit carried 2060 guinea-pig lethal doses, implying an extraordinary resistance to the poison; that is, 240 guinea-pig lethal doses per 200 gm. of rabbit. Doerr and Weinfurter estimated that 2 of their highly toxic rabbits carried from 1700 to 2500 guinea-pig lethal doses. It will be shown in Part VI that, at times, the normal rabbit may carry in its blood 100 of such fatal doses; that is, 10 per 200 gm. rabbit.

It will be seen that the rabbit like the rat is resistant to the poison. Their respective sera, however, as has been shown, differ greatly in the ease with which they are toxified.

SUMMARY

Rat anaphylatoxin, in amounts representing from 1 to 40 lethal doses, invariably caused death in guinea-pigs, the time being essentially the same.

The larger doses gave an intense quiet shock; the autopsy findings in all cases were typical, but with larger doses the petechiæ in the lungs were most pronounced; the blood was always free from clot.

The blood when transferred to a test tube showed delay in coagulation. Slow injection of very large doses gave an almost incoagulable blood.

The in-vitro mixtures of rat anaphylatoxin and normal blood likewise showed retarded coagulation.

The white rat possesses a remarkable immunity against anaphylatoxin; weight for weight it can tolerate more than 100 times as much as the guinea-pig. The dosage with serum reached 10% of the body weight.

A transfusion experiment indicated a rapid disappearance of the anaphylatoxin from the blood.

The heterologous anaphylatoxin, prepared with guinea-pig serum, was tolerated the same as the homologous.

The rabbit like the rat is not affected by large doses of anaphylatoxin and this fact parallels the behavior of rabbits on injection of large doses of the toxic antisheep immune rabbit serum. It is this tolerance of the rabbit for the poison that permits the formation of large amounts of anaphylatoxin during life. Antisheep guinea-pig serum is not toxic since the formation of anaphylatoxin to the extent of a single lethal dose would mean the death of the animal. This tolerance likewise accounts for the known resistance of the rabbit to specific anaphylaxis.